Rilmenidine prevents blood pressure increase in rats with compromised nitric oxide production[1]

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ABSTRACT

AIM: To search tools of high blood pressure in the model of nitric oxide (NO)-defective hypertension, and the study focused on the effect of rilmenidine, agonist of imidazoline receptors, which was suggested to modulate central sympathetic outflow. **METHODS**: Three experimental groups, each consisting of 7 rats, were used: (I) rats with inhibition of NO synthase (NOS) by N^G-nitro-*L*-arginine methyl ester (*L*-NAME) 40 mg·kg^{-1}·d^{-1} for 4 weeks in drinking water, (II) rats with inhibited NOS as in group I , plus agonist of imidazoline receptors rilmenidine 3 mg·kg^{-1}·d^{-1} for 4 weeks by gavage, and (III) control rats. Systolic blood pressure was measured weekly noninvasively. At the end of experiment aortic ring isometric tension was followed, NOS expression (aorta, left ventricle), and NOS activity (left ventricle and brain) were determined. **RESULTS**: In the group I systolic blood pressure increased significantly, aortic ring relaxation to acetylcholine was significantly attenuated. Rilmenidine administered simultaneously with *L*-NAME (group II) prevented the increase of blood pressure which did not differ significantly from control values; aortic ring relaxation to acetylcholine did not differ from control. No change in NOS expression (aorta and left ventricle) was found in groups I and II. Significant decline in NOS activity (left ventricle and brain) was found in groups I and II. **CONCLUSION**: Rilmenidine has a remarkable role in NO-defective hypertension, possibly by inhibiting central sympathetic outflow and by affecting receptors in vascular smooth muscle also. The prime cause of hypertension in this experimental model - the compromised production of NO due to inhibition of NOS - was not affected by rilmenidine.

INTRODUCTION

Several experimental animal models have been developed to recognize and define the mechanisms of human hypertension. Following the discovery of the role of endothelium derived relaxing factor and/or nitric oxide (NO) in vascular smooth muscle tone, the model of NO deficient hypertension in rats was developed in the recent decades[1-3]. The background of the model is inhibition of the activity of NO synthase, which converts arginine to citrulline plus nitric oxide. Compromised production of NO is one tool of vasoconstriction of resistant as well as conduit vessels and thus of high blood pressure[4,5].

Supply of low endogenous nitric oxide by exogenous NO donors nitrites, nitrates, and others, however, do not restore completely the blood pressure to control

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values[6,7]. The deficiency of nitric oxide affects the function of other organs and/or systems directly, and via changes in blood supply also indirectly. Thus further mechanisms have been supposed to be involved in NO-defective hypertension. Morton *et al*[8] and Bernátová *et al*[9] succeeded in declining high blood pressure in NO-deficient hypertensive rats by using inhibitors of angiotensin converting enzyme, pointing out the renin-angiotensin system as another tool.

Having in mind these data, we raised the question concerning the vasomotor centers and nitric oxide. In Wistar normotensive rats, inhibition of NO synthase in posterior hypothalamic area involved in central cardiovascular control, induced an increase in blood pressure[10]. Similarly, Shapoval *et al*[11] demonstrated an increase in blood pressure after NO synthase inhibition in the vasomotor centers of the ventrolateral medulla. Sander *et al*[12] prevented blood pressure increase after NO synthase inhibition by pharmacological peripheral sympathectomy. On the other hand, we found a decrease in density of adrenergic nerve terminals in the myocardium in NO-deficient hypertensive rats after 8 weeks lasting inhibition of NO synthase[13]. Liu *et al*[14] concluded their experiments with the statement that the sympathetic nervous system was not activated or even suppressed in NO defective hypertension.

A novel view on the operation of vasomotor centers was offered by Ernsberger *et al*[15] who revealed that cells of the rostral ventrolateral medulla were endowed with imidazoline receptors. After binding with an agonist, the imidazoline receptors are able to modulate central sympathetic outflow to the periphery. In the present paper, the following issues were addressed: (1) On affecting the imidazoline sensitive area by the exogenous agonist rilmenidine is blood pressure increase prevented in rats after long-term inhibition of NO synthase? (2) Since there are studies considering a possible action of rilmenidine directly in vascular smooth muscle[16], experiments were performed also on isolated vessels. (3) To find out whether rilmenidine interferes with the prime-core cause of high blood pressure in defective NO production, the activity of NO synthase in the left ventricle and the brain, and the expression of endothelial NO synthase in the thoracic aorta and the left ventricle were determined. All three objects were demonstrated to be endowed with NO synthase[17-19].

MATERIALS AND METHODS

Animals Male Wistar rats, 10 weeks of age, weight 301 ± 5 g, were used for the experiments. The rats were housed in individual cages, under 12 h dark-light cycle, constant temperature of 22-24 °C, and with free access to pellet food and water. Procedures and experimental protocol were approved by the Animal Care Committee of the Slovak Academy of Sciences.

Protocol The rats were divided into three groups ($n=7$ in each group):

Group I NO synthase inhibition was induced by administration N^G-nitro-*L*-arginine methyl ester (*L*-NAME). The drug was administered in drinking water in the dose 40 mg/kg per day, for a period of 4 weeks.

Group II The inhibition of NO synthase was performed with the same NO synthase inhibitor *L*-NAME, the same dose and way of administration. Moreover, the animals were administered rilmenidine [(*N*-dicyclopropylmethyl)amino-2-oxazoline, Servier] in the dose of 3 mg/kg dissolved in 0.7 mL tap water, by gavage, daily over the period of 4 weeks.

Group III Age matched rats, housed and fed under the same conditions as group I and II, without any drug treatment, were used as controls.

In each animal systolic blood pressure was measured weekly, noninvasively on the tail artery, using plethysmographic method. Before measuring the animal was maintained in a warm chamber for about 5 min. After the adaptation, blood pressure and heart rate was measured 5 times in 3-5 min intervals consecutively. The mean value was used as representative for statistical evaluation.

After the four-week period of the above treatment the animals were sacrificed. The heart was excised and the left ventricle was prepared, and so was the brain, and both were used to determine the activity of NO synthase. Samples of the left ventricle and thoracic aorta were taken for determination of endothelial NO synthase expression. Thoracic aorta was taken for functional studies *in vitro*.

Response of aortic ring *in vitro* The middle part of the thoracic aorta was cleaned of connective tissue and 3-4 mm long rings were cut with special care to preserve the endothelium. Vascular rings were mounted in tissue baths of 20 mL capacity containing a modified Krebs bicarbonate solution maintained at 37 °C and oxygenated with a mixture of 95 % O_2+5 % CO_2; pH 7.3-7.4. To monitor the isometric tension the aortic rings were vertically fixed between hooks in the incubation bath; the upper part of the ring was connected to the level of a force-displacement transducer Sanborn FT10. The

aortic rings were equilibrated for 1 h under 10 mN resting load, while the solution was changed every 15 min. Aortic rings were precontracted submaximally with phenylephrine (1×10^{-6} mol/L) before being exposed to cumulative doses of acetylcholine. The increase in tension with phenylephrine was considered to be 100 % and the relaxation responses were calculated as percentage of this contraction.

Total NO synthase activity The left ventricle and the brain were homogenized. Total NO synthase activity was determined in crude homogenates by measuring formation of L-[^{3}H]citrulline (L-Cit) from L-[^{3}H]arginine (Amersham, UK) as previously described[20] with minor modifications. Briefly, 50 μL of crude homogenate (7.5 mg of wet tissue) was incubated in the presence of 50 mmol/L Tris HCl, pH 7.4, containing L-[^{3}H]arginine 1 μmol/L (specific activity 5 GBq/mmol), calmodulin 0.5 g/L, β-NADPH 0.5 mmol/L, tetrahydro-biopterin 250 μmol/L, FAD 4 μmol/L, flavin mononucleotide 4 μmol/L, and Ca^{2+} 1 mmol/L, in a total volume of 100 μL. After 10-min incubation at 37 ℃, the reaction was stopped, the samples were centrifuged, and supernatants were applied to 1-mL Dowex 50WX-8 columns (Na^+ form). L-Cit was eluted by 2 mL of water and determined by liquid scintillation counting. NO synthase activity was expressed as picokatals (pkat) per gram of protein.

Endothelial NO synthase expression Samples of left ventricle and thoracic aorta (50 mg of wet tissue) were homogenized in 50 mmol/L Tris HCl, pH 7.4, containing 1 % Triton X-100, leupeptin 1 μmol/L, aprotinin 0.3 μmol/L, PMSF 0.1 mmol/L, and pepstatin 1 mmol/L for Western blot analysis. After centrifugation (15 000×g, 20 min, twice) supernatants were subjected to SDS-PAGE using 8 % gels. After electrophoresis, proteins were transferred to nitrocellulose membranes and probed with a polyclonal rabbit anti-endothelial NO synthase antibody (Santa Cruz Biotechnology, USA). Bound antibodies were detected with a secondary peroxidase-conjugated anti-rabbit antibody (Zymed, Germany). The bands were visualized using the enhanced chemiluminescence system (ECL, Amersham, Buckinghamshire, UK).

Statistics The individual parameters were expressed as mean±SEM. Statistical significance was evaluated using one-way ANOVA and Bonferroni test for unpaired variables. $P<0.05$ was considered statistically significant.

RESULTS

Systolic blood pressure In the group I, significant blood pressure increase was found after L-NAME treatment in the first week: (130.0±3.2) mmHg ($P<0.05$) vs (113.1±1.9) mmHg in controls (group III). Blood pressure increased further and reached the value (153.1±2.8) mmHg ($P<0.01$) at the end of experiment. In the group II, animals treated with L-NAME+rilmenidine, blood pressure did not change during the whole experimental period; its value at the end of experiment (104.7±2.5 mmHg) did not differ from the starting value at the beginning of the experiments (105.9±1.6 mmHg), or from that in control animals 106.4±2.6 mmHg (group III). It differed however significantly from the value of L-NAME-treated animals in the group I, and that from the first week up to the fourth week of experiment when its value was 153.1±2.8 mmHg (Fig 1, $P<0.001$).

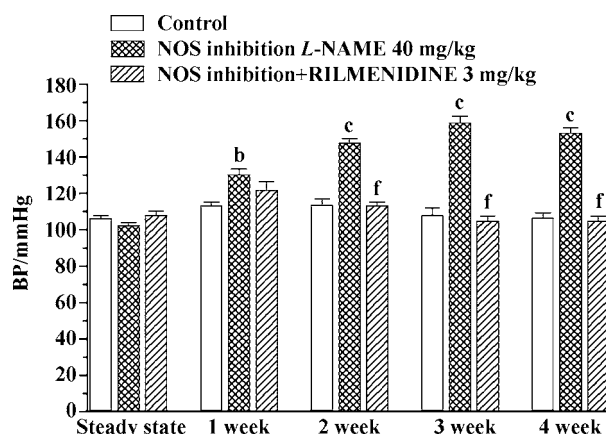**Fig 1. Systolic blood pressure (BP) of rats. n=7. Mean±SEM. ^{b}P<0.05, ^{c}P<0.01 vs control. ^{f}P<0.01 vs L-NAME-treated rats.**

Heart rate No significant alteration in heart rate was found in either of the three groups. A tendency to mild, however, not significant decline of heart rate was seen in the group with NO synthase inhibited (Fig 2).

Heart/body weight ratio In L-NAME-treated hypertensive rats (group I), the value was 3.00±0.15, significantly higher than that in control group (2.62±0.12) ($P<0.01$). In group II, animals treated with L-NAME+rilmenidine, heart/body weight ratio was 2.60±0.06, significantly lower than that in animals treated with L-NAME alone. The value did not differ from the respective value of control animals (Fig 3).
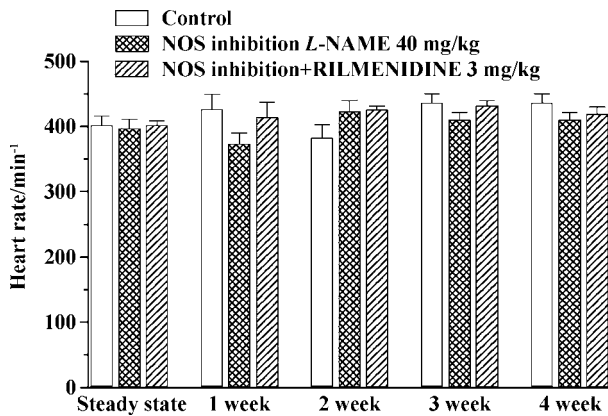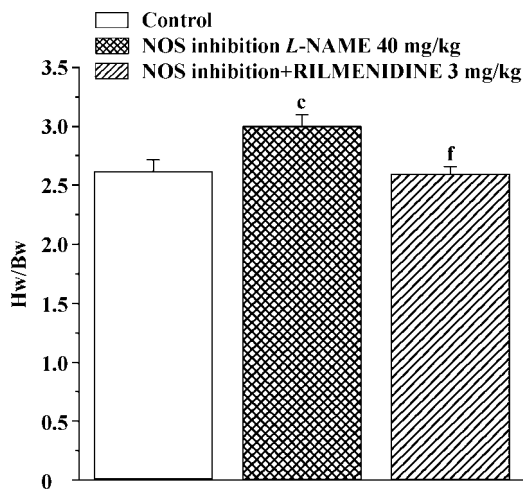
Fig 2. Heart rate of rats. *n*=7. Mean±SEM.

Fig 3. Heart weight/body weight (Hw/Bw) ratio of rats. *n*=7. Mean±SEM. [c]*P*<0.01 *vs* control. [f]*P*<0.01 *vs L*-NAME- treated rats.

Response of aortic ring The magnitude of relaxations was significantly reduced in *L*-NAME-treated rats compared to controls. In *L*-NAME plus rilmenidine-treated animals acetylcholine-induced relaxations were preserved and the magnitude of relaxations did not differ significantly from that of controls (Fig 4).

NO synthase activity In the left ventricle NO synthase activity was 5.60±0.32 pkat/g protein in controls. A lower value of 3.91±0.18 pkat/g protein was found in *L*-NAME-treated animals and also in animals treated with *L*-NAME plus rilmenidine: 3.95±0.20 pkat/g protein (*P*<0.05). A similar display of NO synthase activity values was found in the brain: 9.40±0.71 pkat/g protein in controls, a remarkably lower value of 5.91±0.97 pkat/g protein (*P*<0.05) in *L*-NAME-treated group, and also in animals treated with *L*-NAME plus rilmenidine: 6.85±0.43 pkat/g protein (*P*<0.05, Fig 5).

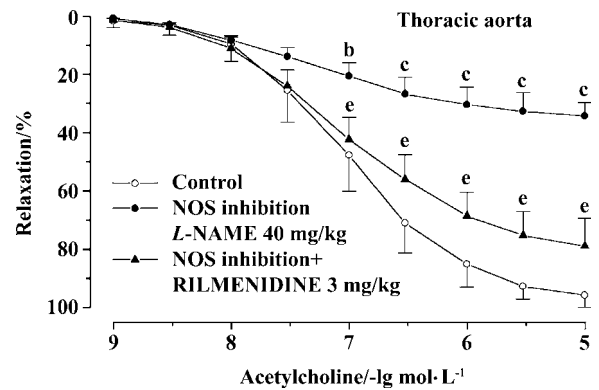**Expression of endothelial NO synthase** No

Fig 4. Concentration-response curves for acetylcholine in aortic ring segments from rats treated for 4 weeks with *L*-NAME (●) and *L*-NAME+rilmenidine (▲), compared with rings from control rats (○). *n*=7. Mean±SEM. [b]*P*<0.05, [c]*P*<0.01 *vs* control. [e]*P*<0.05 *vs L*-NAME treated group.
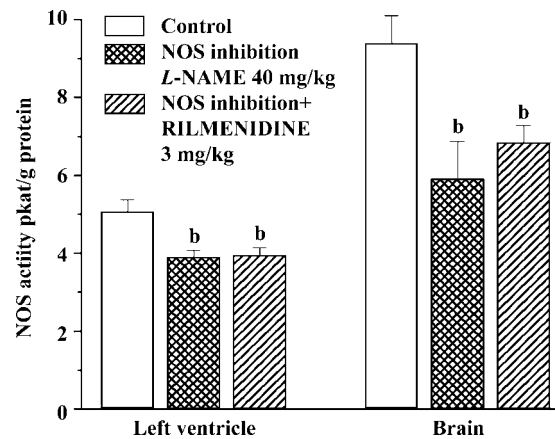

Fig 5. Total NO synthase activity in the left ventricle and the brain. *n*=7. Mean±SEM. [b]*P*<0.05 *vs* control.

changes were found in either the aorta or the left ventricle in *L*-NAME-treated rats and *L*-NAME+rilmenidine treated rats *vs* tissues of control rats (Fig 6).

DISCUSSION

In the group of rats administered NO synthase inhibitor, systolic blood pressure increased immediately in the course of the first week and was maintained at the high level during the 4 weeks. In the rats administered inhibitor of NO synthase activity in drinking water and simultaneously rilmenidine by gavage, the blood pressure increase was unequivocally prevented. No increase was found during the 4-week period of the above treatment.

The consideration that central sympathetic outflow might be involved in NO-defective hypertension
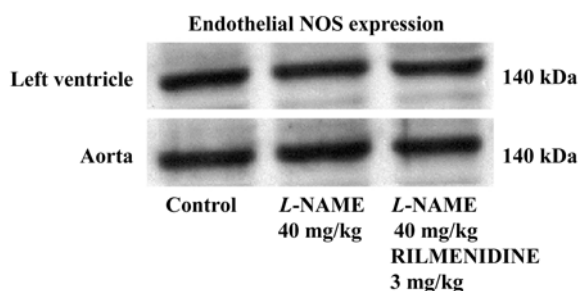
Endothelial NOS expression

Fig 6. Western blot analysis of endothelial NO synthase in the left ventricle and aorta of rats of the three groups studied. A representative blot of four repeats.

has been based on experiments of Bricca *et al*[21] and Gomez *et al*[22] who administered rilmenidine directly into the rostral ventrolateral medulla of normotensive rats and demonstrated a lowering of blood pressure. In anesthetized rabbits hypotension was induced by intra-cisternal administration of rilmenidine even after NO synthase inhibition[23]. This finding is in concert with the view of Molderings *et al*[16] on presence of imidazo-line receptors in central vasomotor areas. The above data together with data presented in our study support the suggestion that the sympathetic nervous system, besides other systems, provides also one tool for main-taining the high blood pressure in NO defective production.

The hypotensive effect of rilmenidine in Wistar rats was demonstrated by ZHANG and JOHNS[24] and Soares de Moura[25]. Soares de Moura[25] found in acute experiments in anaesthetized rats after intravenous ad-ministration of rilmenidine a rapid short-lasting blood pressure increase with subsequent long-term decline of blood pressure. After inhibition of NO synthase the hypotensive response was attenuated mildly. The au-thors admit the relation of rilmenidine and NO-induced vascular tone. The quantitative differences between their experiments and our present results might be ex-plained by different experimental protocol, in particular by the time period of NO synthase inhibition and ad-ministration of rilmenidine.

Since no changes in the heart rate were found in NO-deficient hypertensive rats administered rilmenidine, it seems that the lowering of blood pressure could be ascribed to the vascular component prevalently.

The experiments with isolated thoracic aorta from animals treated with *L*-NAME plus rilmenidine exhib-ited a significant increase in relaxation to acetylcholine in comparison to aortic rings of NO-deficient hyper-

tensive rats. The findings indicate that rilmenidine op-erates probably also on receptors present in areas oth-ers than in the vasomotor centers in the brain, for ex-ample in the thoracic aorta, as shown in the present experiments, and other portions of vascular tree.

Artigues-Varin *et al*[26] did not find any change in rilmenidine-induced relaxation in the isolated mesenteric artery and the artery feeding the gracilis muscle after NO synthase inhibition. Different experimental objects and different experimental protocols might underlie the differences in experimental results.

The present experiments did not clarify the ben-eficial mechanism of rilmenidine on relaxation of the isolated aorta. Nevertheless, one pathway might be ex-cluded according to the present findings. Since in rats treated with *L*-NAME alone, and *L*-NAME plus rilmenidine no difference was found in expression and activity of endothelial NO synthase in the aorta (and left ventricle), nitric oxide may be excluded as the main factor responsible for improvement of aortic relaxation. Binding of rilmenidine to other receptors (α_2-adrener-gic receptors?), or a completely different smooth muscle relaxing mechanism should be considered in designing further experiments.

The question concerning interference of rilmenidine and/or the sympathetic adrenergic system in the prime-core cause of NO-deficient hypertension was addressed by assessing the activity of NO synthase. In rats ad-ministered *L*-NAME, the activity of NO synthase de-creased in the left ventricle and in the brain, as expected. However a decrease of NO synthase activity was found in both tissues also in rats of the experimental group treated with *L*-NAME plus rilmenidine. The results in-dicate that rilmenidine does not influence the activity of NO synthase in the model of NO-defective, *L*-NAME-induced hypertension. In other words, rilmenidine (and/or its modulation of sympathetic nervous system activity) does not affect the metabolic pathway argin-ine →citrulline plus nitric oxide.

The expression of endothelial NO synthase in the aorta and left ventricle of *L*-NAME-treated animals and *L*-NAME plus rilmenidine-treated animals did not differ from the level of NO synthase expression in the re-spective tissues of control animals. Concerning the re-lation of endothelial NO synthase expression and nitric oxide level, the data range from negative feedback[27] to positive feedback relation found in cultures of endothe-lial cells of pulmonary artery[28]. De Gennaro Coronna *et al*[29] found a decrease of endothelial NO synthase

expression in the aorta after NO synthase inhibition due to *L*-NAME administration. The difference between their and our experiments might be due to different doses and time periods of *L*-NAME administration.

Thus the low level of NO, consequently potentially inducing higher vascular smooth muscle tone, operated in cardiovascular system, however, could not be accomplished because it was counterbalanced by rilmenidine modulating both the sympathetic efferentation from vasomotor centers and receptors in vascular smooth muscle.

In conclusion the experiments yielded novel findings that rilmenidine an imidazoline receptor agonist, lowered blood pressure in NO-defective hypertensive rats. The lowering of blood pressure may be induced by lowering sympathetic efferentation from vasomotor centers and also by affecting the imidazoline receptors in vascular smooth muscle. However after rilmenidine treatment the basal cause of NO-defective hypertension (*eg* the low NO-synthase activity and no change in expression of NO-synthase in the brain, heart, and aorta) persisted in spite of lowering blood pressure.

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